

The Neuroblastoma ALK(I1250T) Mutation Is a Kinase-Dead RTK *In Vitro* and *In Vivo*^{1,2}

Christina Schönherr^{*,3}, Kristina Ruuth^{*,3},
Therese Eriksson^{*,3}, Yasuo Yamazaki^{*,3},
Christian Ottmann[†], Valerie Combaret[‡],
Marc Vigny[§], Sattu Kamaraj^{*}, Ruth H. Palmer^{*}
and Bengt Hallberg^{*}

^{*}Department of Molecular Biology, Umeå University, Umeå, Sweden; [†]Chemical Genomics Centre, Dortmund, Germany; [‡]Centre Léon Bérard, FNCLCC, Laboratoire de Recherche Translationnelle, Lyon, France; [§]U839 INSERM/UPMC IFM, Paris, France

Abstract

Activating mutations in the kinase domain of anaplastic lymphoma kinase (ALK) have recently been shown to be an important determinant in the genetics of the childhood tumor neuroblastoma. Here we discuss an in-depth analysis of one of the reported gain-of-function ALK mutations—ALK^{I1250T}—identified in the germ line DNA of one patient. Our analyses were performed in cell culture-based systems and subsequently confirmed in a *Drosophila* model. The results presented here indicate that the germ line ALK^{I1250T} mutation is most probably not a determinant for tumor initiation or progression and, in contrast, seems to generate a kinase-dead mutation in the ALK receptor tyrosine kinase (RTK). Consistent with this, stimulation with agonist ALK antibodies fails to lead to stimulation of ALK^{I1250T} and we were unable to detect tyrosine phosphorylation under any circumstances. In agreement, ALK^{I1250T} is unable to activate downstream signaling pathways or to mediate neurite outgrowth, in contrast to the activated wild-type ALK receptor or the activating ALK^{F1174S} mutant. Identical results were obtained when the ALK^{I1250T} mutant was expressed in a *Drosophila* model, confirming the lack of activity of this mutant ALK RTK. We suggest that the ALK^{I1250T} mutation leads to a kinase-dead ALK RTK, in stark contrast to assumed gain-of-function status, with significant implications for patients reported to carry this particular ALK mutation.

Translational Oncology (2011) 4, 258–265

Introduction

During 2008, anaplastic lymphoma kinase (ALK) receptor tyrosine kinase (RTK) was identified as a familial predisposition gene for the development of neuroblastoma [1]. This study was further supported by four independent reports of additional activating ALK mutations in both familial and somatic neuroblastomas [2–5]. Neuroblastoma is a neural crest-derived embryonal tumor of the postganglionic sympathetic nervous system. Further, it is the most common single solid tumor of childhood with the worst prognosis, constituting almost 6% of diagnosed tumors and more than 9% of all deaths [6]. The origin of these tumors remains unknown in most cases, although a number of familial cases have recently been associated with mutations of the *ALK* gene [1,5]. Neuroblastomas show heterogeneous biologic and clinical features and, whereas a subset may undergo spontaneous differentiation or regression with little or no therapy, the majorities are difficult to cure with current modalities. The

Address all correspondence to: Prof. Bengt Hallberg or Prof. Ruth Palmer, Department of Molecular Biology, Building 6L, Umeå University, Umeå S-901 87, Sweden. E-mail: Bengt.Hallberg@molbiol.umu.se, Ruth.Palmer@ucmp.umu.se

¹This work has been supported by grants from the Swedish Cancer Society (08-0597 to B.H.), the Children's Cancer Foundation (08/084 to B.H. and 08/074 to R.H.P.), the Swedish Research Council (621-2003-3399 to R.H.P.), Lions Cancer Society (to B.H. and R.H.P.), Umeå and the Association for International Cancer Research (08-0177 to R.H.P.). S.K. is a Children's Cancer Foundation fellow (NBCNSPDHEL09/002). R.H.P. is a Swedish Cancer Foundation Research Fellow.

²This article refers to supplementary materials, which are designated by Figures W1 to W3 and are available online at www.transonc.com.

³Joint first authors.

Received 22 March 2011; Revised 22 March 2011; Accepted 24 March 2011

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1944-7124/11

DOI 10.1593/tlo.11139

ALK RTK was first described in the mid-1990s, and aberrant ALK protein activity is now implicated in a range of nonhematopoietic, hematopoietic, as well as neuroendocrine tumors (for review, see Palmer et al. [7]). At this point, there are no clinically approved treatments of aberrant regulated or oncogenic ALK expression, although recent studies provide an optimistic view of Crizotinib, a small ALK and c-Met inhibitor in ALK-positive non-small cell lung cancer and inflammatory myofibroblastic tumor [8–10]. One possible positive offshoot is the potential use of Crizotinib to treat neuroblastoma patients [7].

In this study, we have investigated the described ALK^{I1250T} mutation discovered in a neuroblastoma patient [1]. This mutation had not previously been described in either the SNP database (dbSNP; <http://www.ncbi.nlm.nih.gov/projects/SNP/>) or in the somatic mutation database (COSMIC; <http://www.sanger.ac.uk/genetics/CGP/cosmic/>) [1]. The ALK^{I1250T} mutation was present in the matched germ line DNA, raising the possibility of hereditary predisposition [1]. The original *in silico* analysis predicted ALK^{I1250T} to be an activating mutation in ALK [1]. During 2010, the crystal structure of the ALK kinase domain was described by two groups [11,12], in which the ALK^{I1250T} catalytic loop mutation was described as promoting oncogenesis by altering the substrate binding to become a gain-of-function mutation [11], further reinforcing the notion that ALK^{I1250T} is an activated mutant. However, to our own surprise and in contrast to previous predictions, we clearly observe that rather than being a gain-of-function mutation, the ALK^{I1250T} mutant is actually a kinase-dead RTK.

Materials and Methods

Generation of Human and Mouse ALK Mutant Constructs in Cells and Drosophila

Construction of the mouse 3761 T→C point mutation, corresponding to the mouse I1254T mutation, and the human 3749 T→C, corresponding to the human I1250T mutation was performed using Quick Change Site-Directed mutagenesis kit (Stratagene, [Cedar Creek, TX] according to the manufacturer's instructions) with the following primers: mouse 5'-CACTTTATCCACCGGGATACTGCTGCTA-GAACTG-3' and 5'-CAGTTTCTAGCAGCAGTATCGGCGTG-GATAAAGTG-3' and human 5'-ACCACTTCATCCACCGAGACACTGCTGCCAGAA-3' and 5'-TTCTGGCAGCAGTGTCTCGGTGGATGAAGTGGT-3'. All constructs were confirmed by sequencing from both directions. The mouse ALK^{I1254T} fragment was ligated into the pTTP vector, described in Schönherr et al. [13], resulting in the pTTPmALK I1254T plasmid. Human pcDNA wild-type and F1174S ALK have been described [14]. The human ALKI1250T fragment was ligated into full-length human ALK in both pTTPhALK and pcDNA3(hALK) [14].

Antibodies and Inhibitors

The following antibodies were used: anti-pan-ERK (1:5000) was purchased from BD Transduction Laboratories (Franklin Lakes, NJ), and the anti-p-ERK was from Cell Signaling Technology (Danvers, MA). The activating monoclonal antibodies 46 and 31 (mAb46 and 31) have been described previously [14,15]. Monoclonal Ab no. 153 was produced in the laboratory against the extracellular domain of ALK in similar manners as described [15]. The anti-phosphotyrosine antibody 4G10 was from Upstate Biotech (Lake Placid, NY). Organelle marker antibodies, anti-GRP78/BiP rabbit antibody (ab21685) and anti-GM130 rabbit antibody (ab52649), were obtained from Abcam (Cambridge, United Kingdom).

Cy2-labeled goat antimouse IgG and Cy3-labeled goat antirabbit IgG were from GE Healthcare (Uppsala, Sweden).

The horseradish peroxidase-coupled secondary antibodies goat antirabbit IgG and goat antimouse IgG were from Thermo Scientific (Waltham, MA). The ALK-specific inhibitor NVP-TAE684 has been described previously [13,16].

Cell Lysis, Immunoprecipitation, and Western Blot Analysis

Briefly, both mouse and human PC12ALK^{I1250T} cells were induced with doxycycline and serum starved for 20 hours. PC12mALK^{wt} cells were additionally stimulated with 1 µg/ml of the activating mAb46 for 30 minutes [13,15,17]. Precleared cell lysates were analyzed on SDS/PAGE, followed by immunoblot analysis with the indicated antibodies. ALK downstream activation was detected by p-ERK, and pan-ERK was used to show equal loading. ALK-phosphorylation was detected by the 4G10 antibody. Cell lysis, immunoprecipitation, and immunoblot analysis were performed according to the protocols described in Schönherr et al. [13].

Neurite Outgrowth Assay

Both PC12mALK^{wt} and PC12mALK^{I1254T} cells were seeded sparsely in six-well plates, and ALK expression was induced by doxycycline. PC12mALK^{wt} cells were stimulated with 1 µg/ml mAb46. Quantification of neurite outgrowth in the cells was carried out as described [15]. Experiments were performed in triplicates, and each sample within an experiment was performed in duplicate. For human ALK, 2×10^6 PC12 cells were transfected by electroporation in an Amaxa electroporator (Amaxa, Cologne, Germany) using 0.8 µg of pcDNA3-hALK^{wt} and 0.8 or 1.6 µg of pcDNA3-hALK^{I1250T} and 0.5 µg of pcEGFPN1 (Clontech, Mountain View, CA) and 100 µl of Ingenio electroporation solution (Mirus Bio LCC, Madison, WI). After transfection, cells were transferred to Dulbecco modified Eagle medium (DMEM) supplemented with 7% horse serum and 3% fetal bovine serum, thereafter seeded into 24-well plates together with 1 µg/ml mAb31. Two days after transfection, the fraction of GFP-positive and neurite-carrying cells *versus* GFP-positive cells was estimated under a Zeiss Axiovert 40 CFL microscope (Carl Zeiss, Stockholm, Sweden). To be judged as a neurite-carrying cell, the neurites of the cell had to reach at least twice the length of the diameter of a normal cell body.

Transformation Assay

Low-passage number NIH 3T3 cells (ATCC, Manassas, VA) were transfected with Lipofectamine 2000 according to the manufacturer's protocol. Briefly, 4.5×10^4 cells, seeded the day before into collagen-coated 12-well plates, were transfected for 6 hours with 0.55 µg of pcDNA3 containing hALK^{wt}, hALK^{I1250T}, or hALK^{F1174S} DNA and 1.4 µl of Lipofectamine 2000 in 0.3 ml of Opti-MEM. Twenty-four hours after transfection, three fifths of the cells from each well were transferred to wells in 12-well plates and kept in DMEM (10% fetal calf serum [FCS] and 0.5 mg/ml G418) until the cells reached confluence. Thereafter, cells were kept in DMEM (5% FCS and 0.25 mg/ml G418) for another 10 days.

Cell Culture and Immunofluorescence

CLB-GE neuroblastoma cell line was grown as described [5]. HEK293 cells were grown in DMEM containing 10% heat-inactivated FCS on collagen-coated coverslips. According to the manufacturer's protocol, pcDNA3-ALK^(wt or I1250T) was transfected using Lipofectamine (Invitrogen, Carlsbad, CA). After 24 hours of incubation,

cells were fixed with 4% paraformaldehyde/DMEM for 30 minutes at 37°C and incubated with 10 mM NH₄Cl/phosphate-buffered saline (PBS) for 15 minutes at room temperature. Then, cells were blocked by 5% goat serum and 0.3% Triton X-100 in PBS for 2 hours at room temperature and further incubated with primary antibodies (anti-human ALK mouse mAb153 at 1 µg/ml and anti-GRP78 rabbit antibody diluted 1:200/anti-GM130 rabbit antibody diluted 1:200) in PBS containing 1% bovine serum albumin and 0.3% Triton X-100 at 4°C overnight. After rinsing three times with PBS, cells were incubated with fluorescence-labeled secondary antibodies (Cy2-labeled antimouse IgG and Cy3-labeled antirabbit IgG both at 1:1000 dilution) for 2 hours at room temperature. After rinsing with PBS, coverslips were mounted on slides with Fluoromount G (Southern Biotechnology Associates, Inc, Birmingham, AL). For nonpermeabilized cells, cells were incubated without Triton X-100. Immunostained cells were visualized with a Zeiss fluorescence microscope equipped with an Apotome (Carl Zeiss). Images were acquired and processed by using Zeiss AxioVision 4.8 (Carl Zeiss).

Generation of Human ALK Mutant Transgenic Constructs for *Drosophila melanogaster*

Before ligation of human ALK^{I1250T} from pcDNA3 into the *Drosophila* expression vector pUAST, an 898-base pair fragment preceding the translation start was removed to increase expression efficiency in the *Drosophila* system as described [14], and details are available on request. All three constructs were subsequently subcloned into the *EcoRI*-*NotI* site of the pUAST *Drosophila* vector, and the resulting constructs were verified by DNA sequencing analysis. Transgenic constructs were used for the generation of transgenic fly strains (BestGene, Inc, Chino Hills, CA). The following stocks were used: *w*¹¹¹⁸ (stock number 5905; Bloomington, IN) and pGMR-Gal4 (stock number 9146; Bloomington). The transgenic fly strains *UAS-ALK*^{wt}, *UAS-ALK*^{F1174L}, and *UAS-ALK*^{R1275Q} were generated as described above. Expression in the eye, immunoblot analysis, and fluorescent and electron microscopy of wild-type and mutant ALK proteins were carried out as described in Martinsson et al. [14].

Results

ALK^{I1250T} Is Not a Gain-of-Function RTK in Cell Culture Systems

To investigate the intrinsic activity of the mouse ALK^{I1254T} mutant, which is equivalent to human ALK^{I1250T}, we used an inducible PC12 cell culture system for the clonal expression of both wild-type ALK and mouse ALK^{I1254T} mutant (Figure 1A). On stimulation with an agonist monoclonal antibody (mAb46), the doxycycline-expressed mouse wild-type ALK RTK becomes tyrosine phosphorylated (Figure 1A, top two panels, compare lane 3 with lanes 2 and 1). Further, the stimulated receptor also activates/phosphorylates downstream targets, such as Erk, and the activation/phosphorylation of Erk is not detected when the wild-type ALK receptor is not stimulated (Figure 1A, lower two panels, compare lane 3 with lanes 2 and 1). However, the expression of the putative gain-of-function mouse ALK^{I1254T} shows no activation of Erk and no auto/transphosphorylation activity of the receptor was observed either on induction of expression only or on stimulation of mouse ALK^{I1254T} RTK with mAb46 agonist antibody (Figure 1A, compare lanes 4, 5, and 6).

A sensitive functional readout for activity in PC12 cells is the ability to induce neurite outgrowth. We and others have previously shown that activation of both human and mouse ALK triggers differentiation

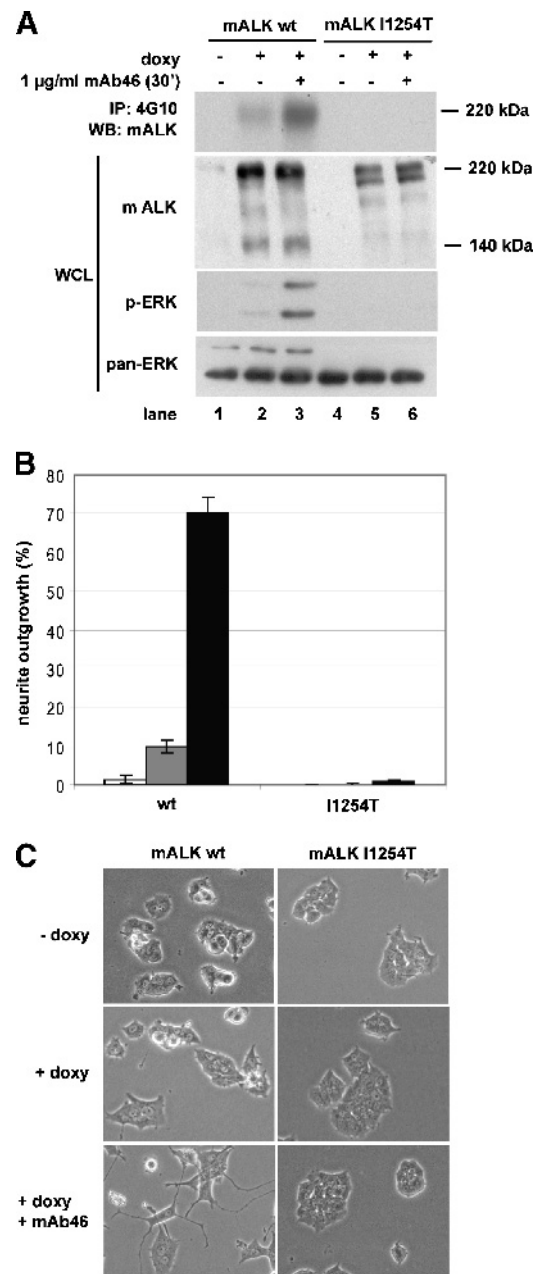


Figure 1. Mouse ALK^{I1254T} mutant is not constitutively active and cannot be activated. (A) To investigate ALK signaling, PC12 cells were induced for expression of either mALK^{wt} or mALK^{I1254T} mutant with 1 and 2 µg/ml doxycycline, respectively, serum starved for 20 hours before stimulation with 1 µg/ml mAb46 for 30 minutes [14]. Precleared cell lysates were analyzed on SDS-PAGE, followed by immunoblot analysis with the indicated antibodies. ALK downstream activation was detected by phospho-ERK and pan-ERK was used to show equal loading. Tyrosine phosphorylation of ALK was visualized with 4G10 beads immunoprecipitation and p-ALK was detected by anti-ALK immunoblot analysis. (B) Neurite outgrowth assay. PC12mALK cells induced for mALK expression were stimulated with 1 µg/ml mAb46. After 48 hours, neurite-bearing cells were scored as described before [14]. The experiment was carried out in triplicate, and each sample within an experiment was performed in duplicate. Statistical significance was obtained by Student's *t* test. *P* values between unstimulated and stimulated mALK^{wt} as well as between stimulated mALK^{wt} and stimulated mALK^{I1254T} were less than .01. (C) Representative figures of neurite outgrowth of PC12mALK cells.

of PC12 cells into sympathetic-like neurons, a process that is characterized by extension of neurites [13]. The expression of both wild-type ALK and ALK^{I1254T} was induced in PC12 cells by the addition of doxycycline (Figure 1, B and C). The activating antibody mAb46 was added simultaneously to cells expressing wild-type and also to cells expressing the ALK^{I1254T} mutant. Images were acquired 48 hours after incubation in the presence of doxycycline and mAb46 (Figure 1C). In agreement with our earlier results, we observed a differentiation of PC12 cells expressing the wild-type ALK on stimulation (Figure 1, B and C). However, we observed that expression of the ALK^{I1254T} mutant is unable to mediate neurite outgrowth within 48 hours, even on cell stimulation with the activating mAb46 (Figure 1, B and C). Importantly, the expression of wild-type ALK in the absence of activating antibodies is unable to mediate neurite outgrowth (Figure 1, B and C). This lack of neurite outgrowth activity observed with ALK^{I1254T} is in contrast to the robust neurite outgrowth achieved on stimulation of mouse ALK and wild-type human ALK (Figure 1, B and C; data not shown).

Mouse and human ALK are very similar, displaying 87% overall homology at the protein level. Indeed, within the kinase domain, they differ at very few amino acids; however, one major difference between mouse and human ALK is at Tyr1604, which is lacking in the mouse ALK protein and has been implicated in tumor progression in human ALK [18]. To confirm our unexpected results with the mouse ALK^{I1254T} mutant, we investigated the human ALK^{I1250T} mutant. On transfection and stimulation of the wild-type human ALK, we observed both an auto/transphosphorylation of the receptor and stimulation of the Erk pathway (Figure 2A, compare lane 4 with 3). Similar to our findings with mouse ALK^{I1254T}, stimulation of the human ALK^{I1250T} neither mediates phosphorylation of the receptor nor could any Erk activity be detected (Figure 2A, compare lanes 6 and 7 with lanes 3 and 4). As a positive control for gain-of-function activity, we used the human ALK^{F1174S} mutation, which is a strongly activating neuroblastoma mutation and which is autophosphorylated and stimulates ERK activity on expression in a ligand-independent manner (Figure 2A, lane 5) [14]. Further, human ALK^{I1250T} was unable to induce neurite outgrowth, in contrast to both human ALK^{F1174S} and the stimulated wild-type ALK receptor (data not shown). Similar experiments with stable clones of PC12 cells were performed with the human wild-type ALK, the activating human ALK^{F1174S}, and human ALK^{I1250T} with identical results (Figure W1). Thus, it is clear that both mouse ALK^{I1254T} mutant and human ALK^{I1250T} display no detectable tyrosine phosphorylation activity. Further, they are unable to activate Erk or to induce neurite outgrowth *in vitro* cell culture systems. We next investigated whether the human ALK^{I1250T} displayed transforming potential. NIH 3T3 cells were transfected with human ALK^{I1250T} in comparison with wild-type ALK and ALK^{F1174S} gain-of-function mutation. The expression of human ALK^{F1174S} mediates foci of transformed cells over the background monolayer, whereas overexpression of the wild-type human ALK receptor and ALK^{I1250T} is unable to mediate foci formation (Figure 2B). Thus, both human ALK^{I1250T} and the equivalent mouse ALK mutant show no intrinsic transforming activity.

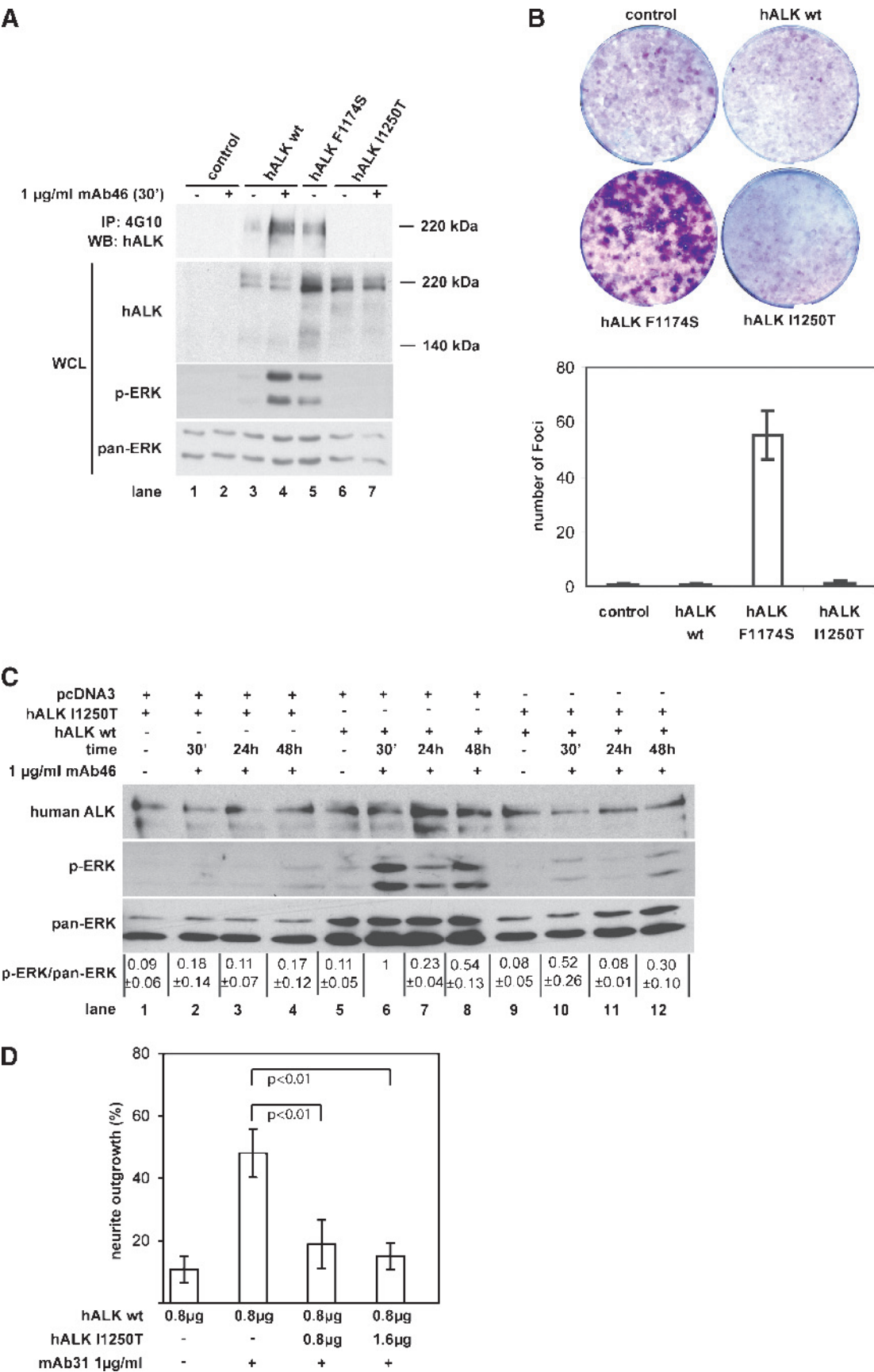
Then we cotransfected wild-type human ALK together with the ALK^{I1250T} mutant. In this analysis, ALK^{I1250T} exhibits a dominant-negative effect, reducing the phosphorylation of ERK on stimulation of wild-type ALK on stimulation. The phosphorylation of Erk is two-fold lower compared with stimulation of Erk in cells only transfected with wild-type ALK over time (Figure 2C, compare lanes 2

and 10 with lane 6 or lanes 3 and 11 with lane 7, or lanes 4 and 12 with lane 8). This result is reinforced by similar findings in four independent experimental setups. First, a reduction of Erk phosphorylation is observed on cotransfection of constitutively active ALK^{F1174S} together with the ALK^{I1250T} mutant (Figure W2A). Second, transfection of FLAG-tagged ALK^{I1250T} into the neuroblastoma cell line CLB-GE, which expresses the ALK^{F1174V} mutation [5], results in a two-fold decrease in ERK phosphorylation compared with untransfected CLB-GE cells (Figure W2B). As a control, the ALK inhibitor NVP-TAE-684 was added to the CLB-GE cell line, abrogating all ALK-mediated ERK phosphorylation (Figure W2B, compare lanes 2 and 4 with lanes 1 and 3). Third, on transient transfection of FLAG-tagged ALK^{I1250T} alone or together with untagged ALK, we observed that FLAG-tagged ALK^{I1250T} is not detectably tyrosine phosphorylated, either on amino acid Y1604 or when analyzed with tyrosine specific antibody 4G10, even on stimulation of wild-type ALK (Figure W2C, compare lane 2 with 4). Finally, using the PC12 cell neurite outgrowth assay to cotransfection either human wild-type ALK alone, or together with ALK^{I1250T}, followed by stimulation of ALK leads to a decrease in neurite outgrowth potential of ALK in the presence of the ALK^{I1250T} mutant (Figure 2D). Similar results are observed if the activating ALK^{F1174S} is transfected together with ALK^{I1250T} (data not shown). Taken together, these six independent experiments argue that the expression of ALK^{I1250T} has the potential to act as a dominant-negative receptor when expressed together with catalytically competent ALK receptors.

Ectopically Expressed Human ALK^{I1250T} Is Inactive in Drosophila melanogaster

Molecular signaling pathways in *Drosophila melanogaster* share conservation of core components with vertebrate pathways. The *Drosophila* ALK RTK mediates activation of the ERK pathway in response to the ligand Jeb in the developing visceral mesoderm, a signaling pathway that is crucial for the formation of the fly gut *in vivo* ([7]). For simplicity, we chose to ectopically express wild-type human UAS-ALK in the *Drosophila* eye using the pGMR-Gal4 driver line, which directs protein expression in the developing photoreceptors of the eye. This provides a sensitive assay for perturbed signaling *in vivo*, and indeed ectopic expression of wild-type human ALK does not result in any obvious phenotype in the adult fly eye (Figure 3B) and is similar to controls (Figure 3A). Expression of the human ALK protein was confirmed both by immunohistochemical and immunoblot analysis of developing eye discs (Figure 3H). Thus, the wild-type ligand-dependent human ALK RTK does not seem to be activated by endogenous *Drosophila* ligands, providing a clean background in which to analyze the activating potential of putative activating mutants of the human ALK as identified in neuroblastoma patients.

Given the clean phenotypic background observed with overexpression of the wild-type human ALK, we proceeded to investigate the *in vivo* signaling potential of the putative activated hALK^{I1250T} mutant in the *Drosophila* system. As would be expected from a gain-of-function ALK mutation, ectopic expression of ALK^{F1174S} leads to the destruction of normal tissue morphology in the developing fly eye (Figure 3D). The destructive effects of ectopic expression of human ALK^{F1174S} in the fly eye are already observed during third instar larval stages, where the organization of ommatidial units in the developing fly eye is clearly disrupted (Figure 3G). However, ectopic expression of activated hALK^{I1250T} mediates no such phenotype and shows similar wild-type phenotype as expression of wild-type human ALK and controls (Figure 3, compare C and F with B and E). Because no human ALK ligand



is present during the development of the *Drosophila* eye, as evidenced by the lack of phenotype observed on the expression of wild-type human ALK, these results confirm that the human ALK^{F1174S} mutant is indeed a ligand-independent activating mutant of the ALK RTK *in vivo*. In stark contrast, the lack of rough eye phenotype observed on the expression of human ALK^{I1250T} suggested that it is not a gain-of-function ligand-independent ALK mutant as previously predicted.

Wild-type ALK and ALK^{I1250T} Are Localized on the Cell Surface

Finally, we asked if the ALK^{I1250T} mutation is localized differently compared with the wild-type receptor. Analysis in the *Drosophila* system (Figure 3, E and F) suggests that, like wild-type hALK, hALK^{I1250T} is localized on the cell membrane. To investigate this further, we asked whether the cellular localization of the ALK^{I1250T} mutation is different from wild-type in mammalian cells. Here, HEK293 cells were transiently transfected with either human ALK^{I1250T} or wild-type ALK. Human ALK^{I1250T} protein is expressed on the plasma membrane in a manner similar to wild-type ALK (Figure 4). Previous reports have shown that ALK protein also localizes intracellularly to structures such as the endoplasmic reticulum and Golgi apparatus [19]. We also find that the ALK^{I1250T} protein shows similar subcellular localization to wild-type ALK, being found in the endoplasmic reticulum (Figure 4, compare A and A' with C and C') and weakly in the Golgi apparatus (data not shown) in addition to plasma membrane localization (Figure 4, compare B with D). Thus, this indicates that both receptors are expressed on the cell surface, providing ALK^{I1250T} with the opportunity to act as a dominant-negative receptor when expressed together with wild-type or gain-of-function ALK receptor.

Discussion

Here we show that the postulated gain-of-function human ALK^{I1250T} shows no detectable biochemical and transforming activity. We show that this is also true for the mouse ALK^{I1254T} mutant. In agreement, the human ALK^{I1250T} mutation is unable to induce a rough eye phenotype in *Drosophila*, in contrast with gain-of-function ALK mutations [14].

Recently, the crystal structure of ALK was reported, which facilitates our understanding of the mechanistic basis of ALK mutations identified in neuroblastoma [11,12]. With the benefit of experimental data demonstrating that ALK^{I1250T} is a kinase inactive mutant, we

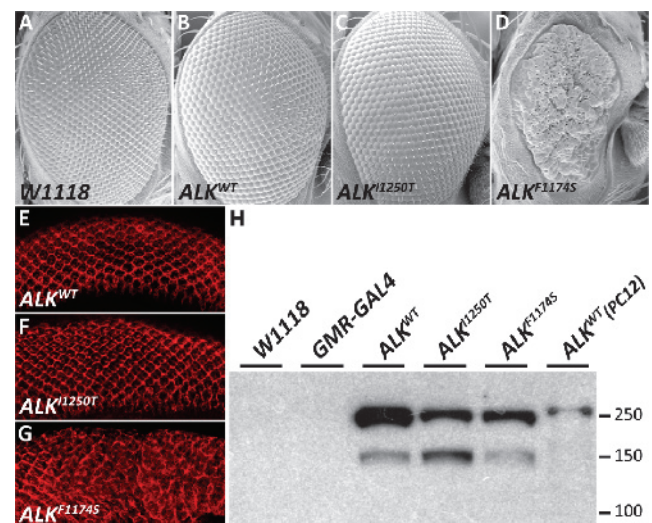


Figure 3. ALK^{I1250T} is not an activating mutation of ALK in *Drosophila*. UAS-ALK transgenes are ectopically expressed in the eye tissue using the eye-specific GMR-GAL4 driver. (A) Wild-type eye. (B) ALK^{wt} expression does not affect eye organization, indicating the absence of an activating ligand in the fly. (C) The expression of ALK^{I1250T} does not cause a rough eye phenotype, indicating that ALK^{I1250T} is not a gain-of-function mutation. (D) ALK^{F1174S} expression causes rough eye phenotype, illustrating that ALK^{F1174S} is a ligand-independent activating mutation. (E–G) Transgene expression is confirmed in larval eye discs by antibody staining against human ALK. (E) Photoreceptor organization is unaffected by expression of ALK^{wt}. (F) ALK^{I1250T} does not affect the photoreceptor pattern. (G) ALK^{F1174S} expression disrupts photoreceptor organization. (H) Western blot analysis of protein lysates of fly heads from flies in which the transgenes have been expressed using the eye tissue-specific driver GMR-GAL4. Protein expression is confirmed by antibody staining against human ALK. Controls are wild-type flies (W1118) and the driver line GMR-GAL4 alone. Positive control is a PC12 cell lysate sample in which hALK transgene expression has been induced by doxycycline.

can exploit the elegant structural studies of Bossi et al. [11] and Lee et al. [12] to suggest a mechanistic explanation. As discussed before, the mutation at amino acid position 1250 is in the catalytic loop of the kinase domain (Figures 5A and W3). Position 1250 of ALK is

Figure 2. Human ALK^{I1250T} mutant is not constitutively active and cannot be activated. (A) To study human ALK signaling, PC12 cells were transiently transfected with 2 μ g of pcDNA3.1 as a control and pcDNA3.1-hALK^{I1250T} as well as 0.8 μ g of pcDNA3-hALK^{wt} and hALK^{F1174S} followed by starvation for 48 hours before stimulating with 1 μ g/ml of the activating mAb46 for 30 minutes [14]. Lysates were analyzed on SDS-PAGE, followed by immunoblot analysis with the indicated antibodies. ALK downstream activation was detected by phospho-ERK and pan-ERK was used to show equal loading. ALK phosphorylation was detected by 4G10-IP, followed by immunoblot analysis with an ALK antibody. (B) Focus formation assay where low-passage NIH 3T3 cells were seeded in six-well plates the day before transfection with 1.5 μ g of each plasmid, as described in Martinsson et al. [14]. Colonies visible by the naked eye were scored in triplicates. Lower panel: Data shown are the average of three independent experiments. Statistical significance was obtained by Student's *t* test. *P* values between hALK^{wt} and hALK^{F1174S} as well as between hALK^{F1174S} and hALK^{I1250T} were less than .01. (C) PC12 cells were transiently transfected with 2 μ g of DNA in total (for cotransfections, 0.8 μ g of pcDNA3.1 as a control, 0.8 μ g of pcDNA3.1-hALK^{wt} and 1.2 μ g of pcDNA3.1-hALK^{I1250T} were applied) as in A. The transfected cells were then serum starved and treated with 1 μ g/ml of the activating mAb46 for the indicated times. Lysis, immunoprecipitation, and immunoblot analysis were performed as described [13]. ALK downstream activation was detected by phospho-ERK, and pan-ERK was used to show equal loading. To show a decrease of hALK^{wt}-mediated ERK phosphorylation by cotransfection with hALK^{I1250T}, the bands for p-ERK and pan-ERK were densitometrically quantified. Relative band intensities from three different experiments are shown as mean \pm SD. (D) PC12 cells were transfected with the indicated amounts of hALK^{wt} and hALK^{I1250T} as described above. The neurite outgrowth assay is similar as to Figure 1B. Statistical significance was obtained by Student's *t* test. *P* values between activated hALK^{wt} and 0.8 μ g of hALK^{I1250T} or 1.6 μ g of hALK^{I1250T} were less than .01.

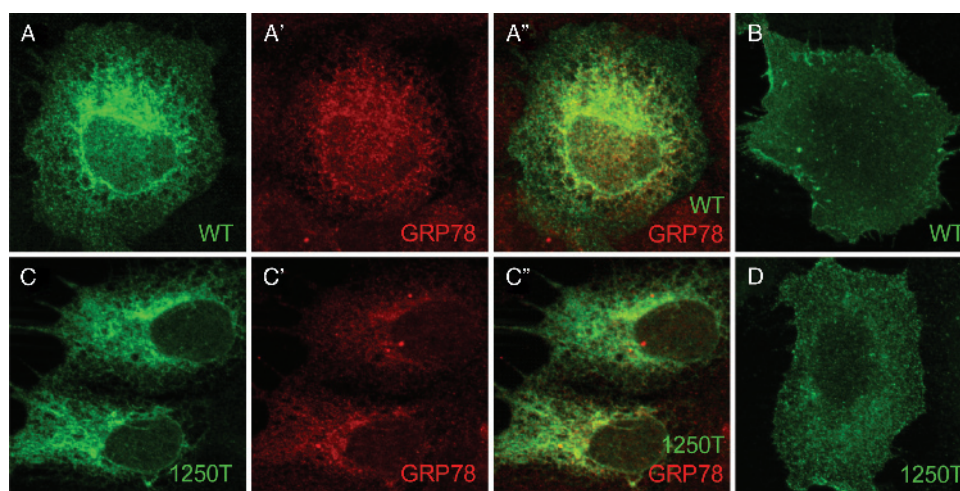


Figure 4. Subcellular localization of the ALK^{I1250T} kinase-dead mutant. Immunofluorescence for hALK wild-type- (A and B) or I1250T- (C and D) expressing HEK293 cells. Cells were immunostained with anti-ALK antibody (A, A' and C, C') and anti-GRP78 antibody as an ER marker (A', A'' and C', C''). (A'' and C'') Merged pictures. (A and C) Detergent permeabilized cells. (B and D) Nonpermeabilized cells. ALK proteins are localized on the cell surface (B and D), the ER (A and C), and weakly at the Golgi (data not shown).

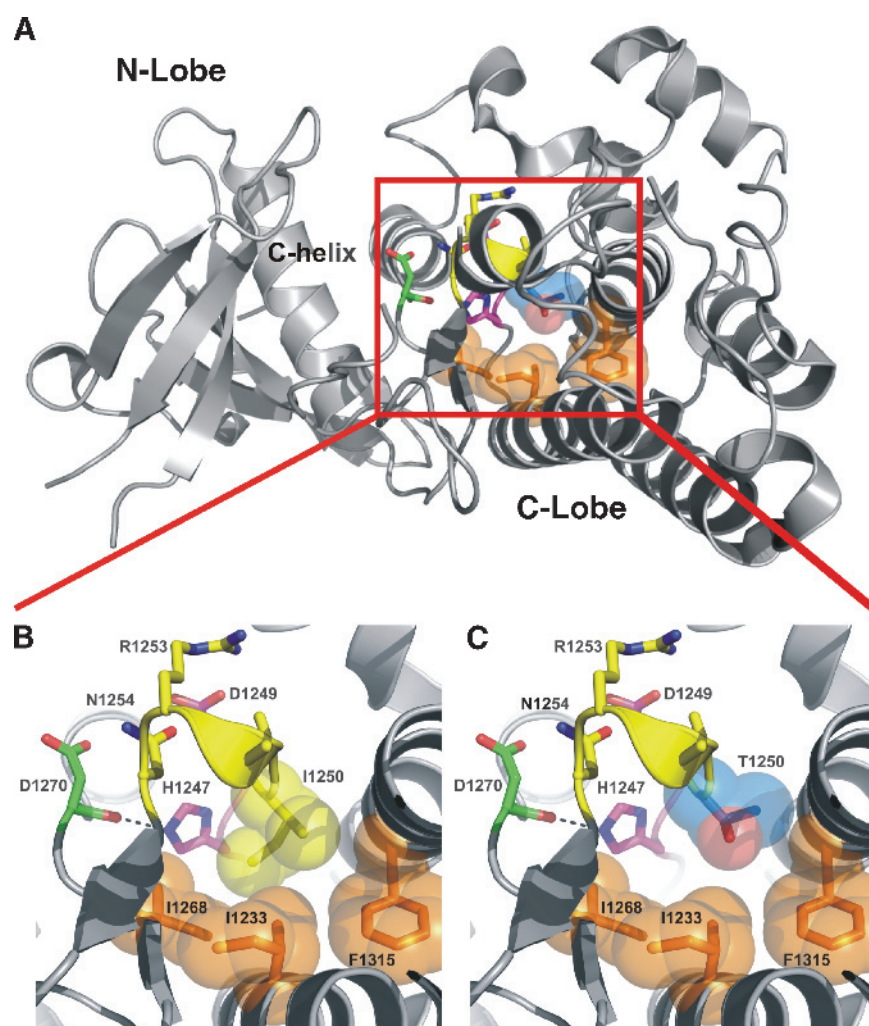


Figure 5. Model of the catalytic domain of ALK containing the I1250T mutation. (A) Model of the N-terminal kinase domain of ALK (PB no. 3LCS). (B) Model of the hydrophobic pocket of human wild-type ALK. (C) Model of the hydrophobic pocket of human ALK with the I1250T mutation.

highly conserved in the active site of protein kinases (Figure 5A) [11,12]. These large, hydrophobic amino acids are part of the conserved catalytic loop sequence HRDI/LAARN, which also influence the hydrophobic spine [20]. The ALK crystal structures have shown that this residue mediates contact with a conserved hydrophobic patch composed of residues from helix 1 and 2 (I1233, I1268, F1315) of the C-lobe (Figure 5B). Hence, the hydrophobic residue of the HRDI/LAARN motif acts to anchor the catalytic loop to establish the correct positioning in respect to the DFG loop and ATP. The I1250T mutation results in a much smaller side chain at this position and introduces a polar functionality (the hydroxyl group of the threonine side chain) into this hydrophobic interface, which probably results in weakening of the hydrophobic contact/anchorage (Figure 5C) presumably leading to the destabilization of the entire active site of ALK. Another possibility for the influence of the I1250T mutation on the catalytic activity of ALK is the close proximity of the side chain of I1250 to H1247. The I1250T mutation could modulate the interaction of the side chain of H1247 with the carbonyl oxygen of D1270 from the DFG motif with a possible destabilizing effect impairing the activation of the kinase. Our structural view on the ALK^{I1250T}, strengthened by experimental evidence is in stark contrast to the earlier descriptions by Mosse et al. [1] and Bossi et al. [11], which describe the ALK^{I1250T} catalytic loop mutation as a gain-of-function mutation promoting oncogenicity.

It is possible that a kinase-dead ALK^{I1250T} might have a biologic/functional activity in the presence of an activated ALK RTK. Indeed, a cooperative tumorigenicity mechanism has recently been described for kinase-dead BRAF in the presence of oncogenic RAS [21]. Further, activation of the kinase-dead ALK receptor could occur through heterologous cross-phosphorylation on tyrosine residues in a ligand-dependent way by the other wild-type ALK [22,23]. However, the data thus far would argue that the ALK^{I1250T} acts in a dominant-negative manner when expressed with active ALK. The implication of the presence of the ALK^{I1250T} mutant for down-regulation *in vivo* is unclear and will require future investigation. However, one could speculate that such a mutation of one copy of the *ALK* locus may have a minor effect in humans because knockouts of ALK in mice have no gross phenotype and are viable and fertile [24] (B.H. and R.P., unpublished results), although they do display increased hippocampal progenitor proliferation and increased performance in hippocampal-associated tasks [25]. Taken together, the results presented here clearly demonstrate that the novel ALK^{I1250T} mutant is not a ligand-independent gain-of-function RTK. Thus, the appearance of the novel ALK^{I1250T} mutant cannot be simply correlated with the development of aggressive neuroblastoma at the patient level and should be considered accordingly.

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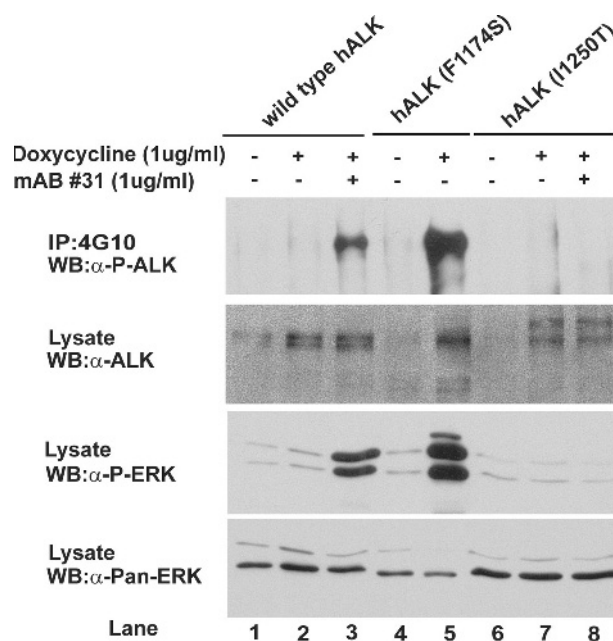


Figure W1. Human ALK^{I1250T} mutant is not constitutively active and cannot be activated. The stable PC12 Tet-on clones PChALK containing either hALK^{wt}, hALK^{F1174S}, or hALK^{I1250T} mutation were generated. To investigate human ALK signaling, 1×10^6 cells were induced for the expression of hALK^{wt}, hALK^{F1174S}, or hALK containing the I1250T mutant with 2 μ g/ml doxycycline. The cells were induced and serum starved for 20 hours before stimulation with 1 μ g/ml mAb31 for 30 minutes. Precleared cell lysates were analyzed on SDS-PAGE, followed by immunoblot analysis with the indicated antibodies. Pan-ERK was used to show equal loading. To detect tyrosine phosphorylation of ALK, the cell lysates were incubated with 4G10 beads (catalog no. 16-101; Upstate), and p-ALK was detected by anti-ALK immunoblot analysis. The mutated hALK fragment was digested with *B/pI-FseI* restriction enzymes and ligated into the *B/pI-FseI* sites (human ALK, position 4143 and 5309) of the full-length human ALK in both pTTPhALK [14]. Stable PC12 Tet-on human ALK and ALK^{F1174S} have been described [13].

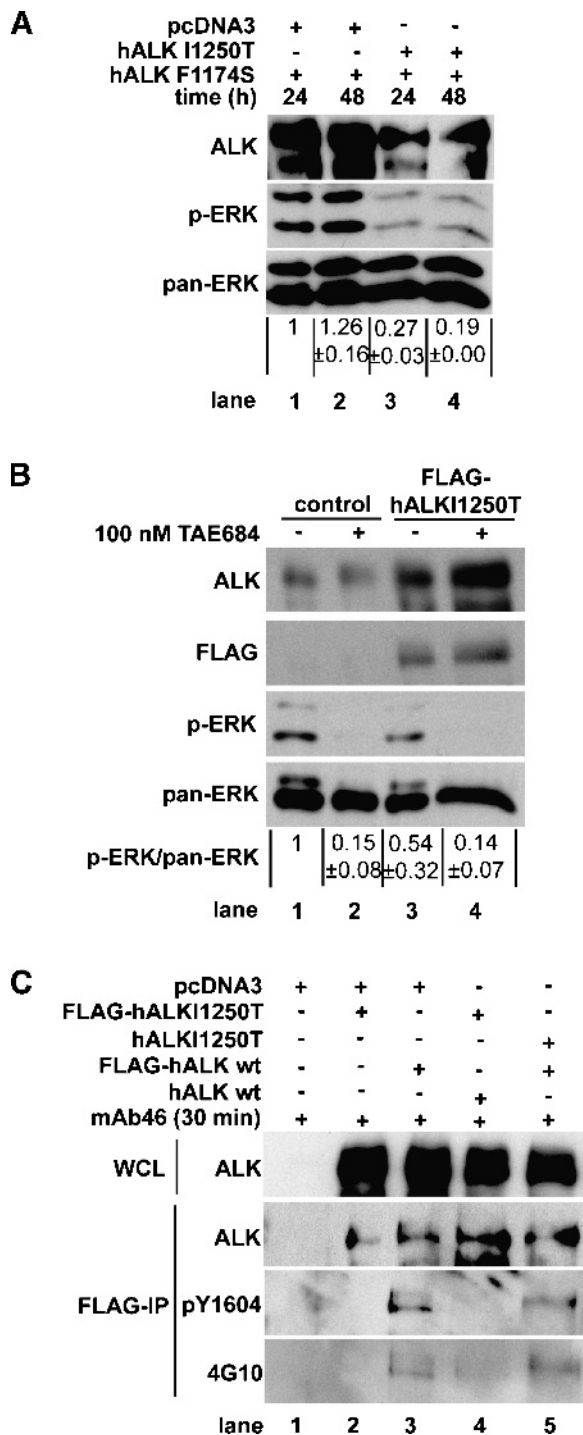


Figure W2. The human ALK^{I1250T} mutant cannot be transactivated by human ALK^{wt}. (A) About 2×10^6 PC12 cells per sample were transiently transfected with 2 μ g of DNA in total (for cotransfections, 0.8 μ g of pcDNA3.1 as a control, 0.8 μ g of pcDNA3.1-hALK^{F1174S}, and 1.2 μ g of pcDNA3.1-hALK^{I1250T} were applied) using Ingenio Electroporation Solution (Mirus Bio LLC, Madison, WI) and the Amaxa Electroporator (Amaxa Biosystems) according to the manufacturers' protocol. The transfection efficiency was approximately 60% to 80%. The transfected cells were then serum starved and harvested after 24 and 48 hours, respectively. Precleared cell lysates were analyzed on SDS-PAGE, followed by immunoblot analysis with the indicated antibodies. ALK downstream activation was detected by p-ERK, and pan-ERK was used to show equal loading. To show a decrease of hALK^{wt}-mediated ERK phosphorylation by cotransfection with hALK^{I1250T}, the bands for p-ERK and pan-ERK were densitometrically quantified. Relative band intensities from three different experiments are shown as mean \pm SD. (B) About 2×10^5 cells of the neuroblastoma cell line CLB-GE were transiently transfected with FLAG-hALK^{I1250T} using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were serum starved and treated with 100 nM NVP-TAE684 for 24 hours. Precleared cell lysates were analyzed on SDS-PAGE, followed by immunoblot analysis with the indicated antibodies. ALK downstream activation was detected by p-ERK, and pan-ERK was used to show equal loading. To show a decrease of hALK^{wt}-mediated ERK-phosphorylation by cotransfection with hALK^{I1250T}, the bands for p-ERK and pan-ERK were densitometrically quantified. Relative band intensities from three different experiments are shown as mean \pm SD. (C) About 2×10^6 PC12 cells per sample were transiently transfected with 2 μ g of pcDNA3.1 as a control, 1.2 μ g of pcDNA3.1-hALK^{I1250T} or FLAG-hALK^{I1250T}, as well as 0.8 μ g of pcDNA3.1-hALK^{wt} or FLAG-hALK^{wt} using Ingenio Electroporation Solution (Mirus BIO LLC) and the Amaxa Electroporator (Amaxa Biosystems) according to the manufacturers' protocol. The transfected cells were then serum starved for 24 hours before stimulation with 1 μ g/ml of the activating mAb46 for 30 minutes. The FLAG-tagged human ALK variants were immunoprecipitated by FLAG antibody (Sigma, St Louis, MO), followed by SDS-PAGE analysis and immunoblot analysis with the indicated antibodies. ALK activation and tyrosine phosphorylation were detected by pY1604 and 4G10 antibodies.

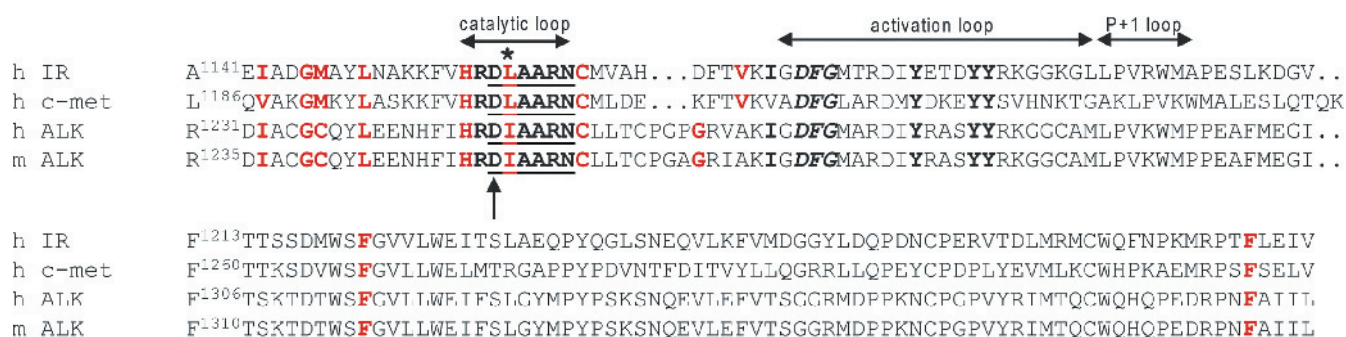


Figure W3. Sequence alignment of catalytic domains of RTKs. Sequence alignment of some catalytic domains of human insulin receptor (Swiss-Prot P096213), human c-Met Receptor (Swiss-Prot P08581), and both human (Swiss-Prot Q9UM73) and mouse ALK (Swiss-Prot P97793). The HRD motif, the autophosphorylated tyrosines of the YxxxYY motif and the hinge region (I before the DFG motif), are marked in bold. The DL/IAARN motif is highlighted in bold and underlined. The DFG motif in the activation loop is in italic and the position 1250 for the I→T mutation is marked by an asterisk. The conserved catalytic D is marked by an arrow. The amino acids forming the hydrophobic pocket are highlighted in red.